

**This Page Is Inserted by IFW Operations
and is not a part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- **BLACK BORDERS**
- **TEXT CUT OFF AT TOP, BOTTOM OR SIDES**
- **FADED TEXT**
- **ILLEGIBLE TEXT**
- **SKEWED/SLANTED IMAGES**
- **COLORED PHOTOS**
- **BLACK OR VERY BLACK AND WHITE DARK PHOTOS**
- **GRAY SCALE DOCUMENTS**

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

THIS PAGE BLANK (USPTO)

PCT

WORLD INTELLIGENCE



WO 9604001A1

INTERNATIONAL APPLICATION PUBLISHED

(51) International Patent Classification ⁶ : A61K 38/00, 38/18, 31/70, 39/44, 39/385, 39/395		(11) International Publication Number: WO 96/04001
A1		(43) International Publication Date: 15 February 1996 (15.02.96)
(21) International Application Number: PCT/US95/09870		(81) Designated States: AU, CA, HU, JP, NO, European patent AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
(22) International Filing Date: 4 August 1995 (04.08.95)		
(30) Priority Data: 08/286,327 5 August 1994 (05.08.94) US 08/487,693 7 June 1995 (07.06.95) US		
(71) Applicant: MOLECULAR/STRUCTURAL BIOTECHNOLOGIES, INC. [US/US]; Suite 700, 3 Bethesda Metro Center, Bethesda, MD 20814 (US).		
(72) Inventors: KATZ, Robert; 16700 Sioux Lane, Gaithersburg, MD 20878 (US). TOMOAI-A-COTISEL, Maria; 254 Congressional Lane #102, Rockville, MD 20852 (US).		
(74) Agent: REA, Teresa, Stanek; Burns, Doane, Swecker & Mathis, P.O. Box 1404, Alexandria, VA 22313-1404 (US).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: SITE-SPECIFIC BIOMOLECULAR COMPLEXES		
(57) Abstract <p>The present invention describes site-specific biomolecular complexes comprising a therapeutic, prophylactic and diagnostic agent, called also biologically active molecule and an omega-3 fatty acid and derivatives thereof. The complexes are further covalently bonded with cationic carriers and permeabilizer peptides for delivery across the blood-brain barrier and with targeting moieties for uptake by target brain cells. Said complexes are particularly useful for delivery of a biologically active agent to the glial tissue of the brain as well as to the cortical, cholinergic and adrenergic neurons. The preferred therapeutic complexes or conjugates comprise an omega-3 fatty acid such as alpha-linolenic acid, eicosapentaenoic acid or docosahexaenoic acid and derivatives thereof.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

-1-

SITE-SPECIFIC BIOMOLECULAR COMPLEXES

FIELD OF THE INVENTION

The present invention relates to site-specific biomolecular lipophilic complexes, also called conjugates or transport vectors. These complexes comprise an omega-3 fatty acid and derivatives thereof, and a therapeutic, prophylactic, diagnostic or a research agent. Further, said complex is conjugated with cationic macromolecular carriers to enhance its passage from the blood circulation to the brain. The resultant conjugates are specific for sustained release delivery of said agents to the central nervous system (CNS), particularly to the neurons of the cerebral cortex, hippocampus and corpus striatum (e.g., cholinergic and adrenergic neurons) and to the glial tissue (neuroglial cells, including, astrocytes and macroglial cells) in animals and humans.

In addition, targeting or internalizing moieties, such as neurotoxins and fragments thereof, are attached to said complex to facilitate its uptake by the target brain cells.

Also, disclosed are pharmaceutical compositions containing poly-unsaturated site-specific complexes/conjugates for the treatment of a wide variety of diseases causing to severe deterioration of the central nervous system, e.g., dementias, neurodegenerative disorders, neurological diseases, malignant brain tumors, inborn errors of metabolism (i.e., lysosomal storage disorders), and the like.

The specification and claims that follow cite numerous patents and publications and their content whether for background or experimental purposes are incorporated by reference herein.

BACKGROUND

During the last two decades, various drug delivery systems have been designed by using carriers such as proteins, peptides, polysaccharides, synthetic polymers, colloidal particles (i.e., liposomes, vesicles or micelles), microemulsions, microspheres and nanoparticles. These carriers, which contain entrapped

-2-

pharmaceutically useful agents, are intended to achieve controlled cell-specific or tissue-specific drug release.

Further efforts and research are being directed to develop and design novel systems of specific delivery to a target cell or tissue for the agents that cross
5 biological barriers at relatively low rates. The purpose of this invention is to present a method that improves the transport and delivery characteristics of an agent molecule to a desired location, in the central nervous system, thus increasing its bioavailability.

The term "agent" as used herein relates to therapeutic, prophylactic and diagnostic compounds. These compounds are biologically active with beneficial
10 effects in both animals and humans. Agents include lysosomal enzymes such as ceramidase, glucocerebrosidase, beta-galactosidase, beta-hexosaminidase A, beta-hexosaminidase A & B, galactosylceramidase, arylsulfatase A, sphingomyelinase, alpha-galactosidase B, aspartylglycosaminidase, alpha-L-fucosidase, iduronate
15 sulfatase, alpha-L-iduronidase, glcNAc-6-sulfatase, beta-glucuronidase, their recombinant analogs and their derivatives. Also included are serum proteins namely immunoglobulins, interleukins, interferons, hormones, such as insulin, parathyroid hormone, pigmentary hormone, thyroid-stimulating hormones, tissue plasminogen activator, nerve growth factors, peptidases or proteases, nucleic acids and derivatives thereof, nucleotides, oligonucleotides, antisense oligonucleotide analogs, genes,
20 transfected cells, biological vectors, cloning vectors and expression vectors. Neurotoxins or their non-toxic peptide fragments, diagnostic and research reagents are also included.

Complexes or conjugates of the above macromolecular agents with omega-3 fatty acid molecules and their derivatives are synthesized to improve transport
25 properties, rates of distribution throughout the brain and targeted delivery of the agent to certain specific sites.

In order to exert desired therapeutic or prophylactic effects, agents must reach brain cells and tissue. Intravenous administration will require their passage from the blood to the brain by crossing the microcapillary membranes of the cerebrovascular
30 endothelium also called the blood-brain barrier or BBB.

-3-

Briefly, the blood-brain barrier (BBB) [Johansson, Progress in Brain Research, 91: 171-175 (1992); Ermisch, Progress in Brain Research, 91: 155-161 (1992); Schlosshauer, BioEssays, 15(5): 341-346 (1993)] is formed by a monolayer of tightly connected microvascular endothelial cells with anionic charges. This layer separates two fluid-containing compartments: the blood plasma (BP) and extracellular fluid (ECF) of the brain parenchyma, and is surrounded by astroglial cells of the brain. One of the main functions of the BBB is to regulate the transfer of components between the BP and the ECF. The BBB limits free passage of most agent molecules from the blood to the brain cells.

In general, large molecules of high polarity, such as peptides, proteins, (e.g., enzymes, growth factors and their conjugates, oligonucleotides, genetic vectors and others) do not cross the BBB. Therefore poor agent delivery to the CNS limits the applicability of such macromolecules for the treatment of neurodegenerative disorders and neurological diseases.

Several delivery approaches of therapeutic agents to the brain circumvent the BBB. Such approaches utilize intrathecal injections, surgical implants [Ommaya, Cancer Drug Delivery, 1(2): 169-178 (1984) and U.S. Pat. No. 5,222,982,] and interstitial infusion [Bobo et al., Proc. Natl. Acad. Sci. USA, 91: 2076-2080 (1994)]. These strategies deliver an agent to the CNS by direct administration into the cerebrospinal fluid (CSF) or into the brain parenchyma (ECF).

Drug delivery to the central nervous system through the cerebrospinal fluid is achieved by means of a subdurally implantable device named after its inventor the "Ommaya reservoir". The reservoir is used mostly for localized post-operative delivery of chemotherapeutic agents in cancers. The drug is injected into the device and subsequently released into the cerebrospinal fluid surrounding the brain. It can be directed toward specific areas of exposed brain tissue which then adsorb the drug. This adsorption is limited since the drug does not travel freely. A modified device developed by Ayub Ommaya, whereby the reservoir is implanted in the abdominal cavity and the injected drug is transported by cerebrospinal fluid (taken from and returned to the spine) all the way to the ventricular space of the brain, is used for agent administration. Through omega-3 derivatization, site-specific biomolecular

complexes of this invention are overcoming the limited adsorption and movement of the agent through brain tissue. Prior art is silent on agents capable of site-directed penetration of brain tissue from the cerebrospinal fluid.

Diffusion of macromolecules to various areas of the brain by convection-enhanced delivery is another method of administration circumventing the BBB. This method consists of: a) Creating a pressure gradient during interstitial infusion into white matter to generate increased flow through the brain interstitium (convection supplementing simple diffusion); b) Maintaining the pressure gradient over a lengthy period of time (24 hours to 48 hours) to allow radial penetration of the migrating compounds (such as: neurotrophic factors, antibodies, growth factors, genetic vectors, enzymes, etc.) into the gray matter; and c) Increasing drug concentrations by orders of magnitude over systemic levels. Through their direct infusion into the brain parenchyma, the site-specific biomolecular complexes of this invention are instrumental in delivering the agent to neuronal or glial cells, as needed, and be retained by these cells. Moreover, the site-specific complexes containing neuronal targeting or internalization moieties are capable of penetrating the neuronal membrane and internalizing the agent. Prior art is silent on such site-specific delivery of macromolecular agents to specific sites in the central nervous system such as cortical and hippocampal neurons.

Another strategy to improve agent delivery to the CNS is by increasing the agent absorption (adsorption and transport) through the BBB and their uptake by the cells [Broadwell, *Acta Neuropathol.*, 79: 117-128 (1989); Pardridge et al., *J. Pharmacol. Experim. Therapeutics*, 255(2): 893-899 (1990); Banks et al., *Progress in Brain Research*, 91: 139-148 (1992); Pardridge, *Fuel Homeostasis and the Nervous System*, Edited by Vranic et al., Plenum Press, New York, 43-53 (1991)]. The passage of agents through the BBB to the brain can be enhanced by improving either the permeability of the agent itself or by altering the characteristics of the BBB. Thus, the passage of the agent can be facilitated by increasing its lipid solubility through chemical modification, and/or by its coupling to a cationic carrier, or still by its covalent coupling to a peptide vector capable of transporting the agent through the

-5-

BBB. Peptide transport vectors are also known as BBB permeabilizer compounds [U.S. Pat. No. 5,268,164].

Therefore, another major objective of this invention is to synthesize a site-specific macromolecule with lipophilic properties. The resulting complex or conjugate, which comprises an agent, one or more lipophilic moieties and a directing moiety, is targeted to specific cells in the brain. The lipophilic moiety is either alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) or derivatives thereof (e.g., alpha-linolenoyl-aminoethanol, N-eicosapentaenoyl-aminoethanol, N-docosahexaenoyl-amino ethanol, or alpha linolenoyl-ethanolamine, eicosapentaenoyl-ethanolamine, docosahexaenoyl-ethanolamine, or lipids of all three omega-3 acids).

There is significant transport of poly-unsaturated fatty acids of the omega-3 series (PUFA, omega-3) across the BBB that is possibly mediated by a specific transporter and/or a receptor or simply by the transcytosis process. Prior art discloses that the BBB can target the release of omega-3 fatty acids into the brain and thus aid in the enrichment of these essential fatty acids in the brain [Yerram et al., J. Lipid Research, 30: 1747-1757 (1989); Moore et al., J. Neurochem., 55(2): 391-402 (1990); Moore et al. J. Neurochem., 56(2):518-524 (1991)]. Also, prior art indicates that the microvascular endothelium (the BBB) and astrocytes, and not neurons, are responsible for the elongation and desaturation of alpha-linolenic acid and eicosapentaenoic acids respectively in the rodent brain (Moore et al., *ibid*). Moreover, astrocytes are apparently responsible for the elongation and desaturation of EPA into docosahexaenoic acid (DHA, or C22:6, omega-3), the release of the latter into the extracellular space or transfer to neurons. Whichever the mechanism, the DHA ends up in the neuronal membrane in form of phospholipids. If the DHA is synthesized in the liver or administered intravenously, then it apparently crosses the BBB to the brain. Therefore, the DHA enrichment in the brain may result from three important roles of the BBB: (1) to take up ALA from the blood, transform it into EPA and eliminate the latter into the perivascular glial cells of the brain, (2) take up DHA from the blood and release it into the brain and (3) to block the egress of DHA from the brain. The latter is probably responsible for the relatively high DHA

content of brain phosphotriglycerides when compared to other polyunsaturated fatty acids (N. Salem et al., in Health Effects of Polyunsaturated Fatty Acids in Seafoods. A.P. Simopoulos, ed. Academic Press, N.Y., 1986, p. 263-317 and G.Y. Sun and L.L. Foudin, in "Phospholipids in Nervous Tissue", J.E. Eichenberg, ed. John Wiley & Sons, N.Y. 1985, p. 95-115. Therefore, EPA directs the agent to the glia and DHA directs the agent to cortical, cholinergic and adrenergic neurons. ALA will reach the microvascular endothelium where it will be transformed into EPA. The use of lipophilic moieties could further protect the agent from enzymatic degradation during its passage across the BBB.

10 Prior art shows, for example, conjugates of proteins with water soluble polymers which have been manufactured and used for pharmaceutical purposes. U.S. Pat. No. 4,935,465 teaches the attachment of one or more polymer molecules to a protein. However, attachment of polymers to proteins, particularly to enzymes which act on low molecular weight substrates, retards enzyme clearance and decreases
15 enzyme antigenicity. When the enzyme acts on a macromolecular substrate or on a cell-bound substrate, the enzyme activity of the conjugate could be diminished.

Other examples [U.S. Pat. No. 4,701,521, and U.S. Pat. No. 4,847,240] describe a method of covalently bonding an agent to a cationic macromolecular carrier which enters into the cells at relatively higher rates. These patents teach enhancement
20 in cellular uptake of bio-molecules into the cells when covalently bonded to cationic resins.

U.S. Pat. No. 4,046,722 discloses anti-cancer drugs covalently bonded to cationic polymers for the purpose of directing them to cells bearing specific antigens. The polymeric carriers have molecular weights of about 5,000 to 500,000.

25 Further work involving covalent bonding of an agent to a cationic polymer through an acid-sensitive intermediate (called also spacer) molecule, is described in U.S. Pat. No. 4,631,190 and U.S. Pat. No. 5,144,011. Various spacer molecules, such as cis-aconitic acid, are covalently linked to the agent and to the polymeric carrier. They control the release of the agent from the macromolecular carrier when
30 subjected to a mild increase in acidity, such as probably occurs within a lysosome of the cell. The drug can be selectively hydrolyzed from the molecular conjugate and

-7-

released in the cell in its unmodified and active form. Molecular conjugates are transported to lysosomes, where they are metabolized under the action of lysosomal enzymes at a substantially more acidic pH than other compartments or fluids within a cell or body. The pH of a lysosome is shown to be about 4.8, while during the initial stage of the conjugate digestion is possibly 3.8.

U.S. Pat. No. 5,308,701 discloses a method for encapsulating proteins within a synthetic cationic poly-L-lysine, crosslinked with multivalent ions of the opposite charge, to form a hydrolytically stable gel.

In general, both natural and synthetic polymers have been used as drug carriers. Synthetic cationic polymers are more suitable drug carriers than natural polymers because their structure can be systematically altered in a defined way and therefore, it is possible to design them to suit biological requirements such as penetration through the blood-brain barrier.

In the present invention, the polymeric cationic carrier system used to facilitate the crossing of the blood-brain barrier comprises poly-L-lysine (PLL). PLL is a biocompatible, hydrophilic polymer with very thoroughly studied chemical and biological properties. Other cationic polyamino acids such as polyarginine and polyornithine are within the scope of this invention. These polyamino acids are covalently attached to a biopolymeric agent conjugated with omega-3 fatty acid molecules or other polymeric protecting groups (e.g., polyethylene glycol). In addition, we have such complexes/conjugates covalently attached to omega-3 molecules for improved binding by the microvascular endothelial cell membrane and targeting to specific areas of the brain. A nontoxic fragment of a neurotoxin is further attached to assure targeting and internalization by neurons once the neuronal target is reached. Spacers can be inserted between the components of the vehicle as shown in the description of the preferred embodiments.

When the biopolymeric agent is an enzyme molecule, one or more fatty acid moieties (e.g., ALA, EPA or DHA) can be attached to the enzyme by reacting an imidate activated enzyme with the respective fatty acid. The number of attached moieties is limited only by the number of available $-NH_2$ groups of the enzyme.

-8-

The prior art is silent on lipophilic macromolecular conjugates, potentially containing polycationic amino acids (targeting lipophilic macromolecular vehicles), used to deliver therapeutic or diagnostic agents across the BBB selectively to specific areas of the brain.

5

OBJECTIVES OF THE INVENTION

A major object of the present invention is to synthesize site-specific biomolecular complexes for the selective transport of a therapeutic, prophylactic and diagnostic agent to the target brain cells. The lipophilic components of the macromolecular complex or conjugate are biocompatible with tissue components. The resultant lipophilic (agent:carrier) complexes, conjugates or transport vectors,
10 containing highly unsaturated omega-3 moieties, assure selective uptake by similar lipid compositions of the cell membranes. The targeting complexes are further specific for agent delivery through receptor pathways to neuronal membranes or to astroglial cells.

15

Preferred features of the present invention are the synthesis and use of lipophilic targeting complexes to deliver the "required agent dose" in vivo to specific areas of the brain, in controlled therapeutic levels by intravenous infusion, intrathecal injection into the cerebrospinal fluid (CSF), or direct infusion into the brain interstitial fluid (BIF).

20

SUMMARY OF THE INVENTION

The instant invention describes a series of site-specific therapeutic, prophylactic or diagnostic complexes comprising a biologically active molecule (agent), one or more residues of an omega-3 fatty acid or derivatives thereof, a polyamino acid as a cationic carrier and a neuron-targeting, internalizing moiety. By
25 complex or conjugate is meant a molecule that is either held together by van der Waal's and electrostatic interactions or by covalent bonds. The preferred omega-3 fatty acids are eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and alpha-linolenic acid (ALA). It should be noted that the specific bonding in the complex will be dictated by the specific chemistries used to form the complex of the invention.

The therapeutic complexes of the present invention are particularly useful for treating diseases that occur in the glial tissue of the brain and diseases that occur in the cortical, cholinergic and adrenergic neurons of the central nervous system.

The biologically active agent is selected from the group consisting of amino acids, peptides, poly peptides, proteins, fusion proteins, polyethylene glycol-derivatized proteins, enzymes, hormones, growth factors, antigens, antibodies, nucleic acids and derivatives thereof, DNA- and RNA-segments, oligonucleotides, antisense oligonucleotide analogs, nucleotides and derivatives thereof, genes, transfected cells, biological vectors, cloning vectors, expression vectors, neurotoxins and fragments thereof, and diagnostic agents. The biological agent can further be derivatized with peptide residues called permeabilizer peptides for facilitated crossing of the blood-brain barrier.

The site-specific complex of the present invention may optionally further comprise a polyamino acid such as polylysine, polyarginine, polyornithine and polyasparagine. The preferred polyamino acid is a polylysine having 1-50 lysine residues and preferably from 10 to 25 lysine residues. These polyamino acids can further be derivatized with omega-3 polyunsaturated fatty acids (ALA, EPA or DHA) and permeabilizer peptides for facilitated crossing of the blood-brain barrier.

The site-specific complex of the present invention may optionally further incorporate a neuronal targeting and/or internalizing moiety selected from the group consisting of the C-fragment of tetanus toxin (TTC), of nontoxic fragment of alpha-bungarotoxin (ABT), other neuron-specific toxins and their nontoxic fragments that still retain their directing and internalizing capacity, and nerve growth factor (NGF).

The instant invention is further inclusive of methods for the site-directed delivery of drugs to treat diseases of the glial tissue of a mammalian brain by intrathecally administering to the afflicted mammal a therapeutically effective amount of a biologically active agent, called also drug, that has been derivatized with eicosapentaenoic acid. When it is desired to treat diseases of the cortical, cholinergic and adrenergic neurons the active agents are derivatized with docosahexaenoic acid.

The instant invention is further inclusive of methods for the site-directed delivery of the above complex/conjugate by direct infusion into the interstitial matter of the brain of the afflicted mammal.

5 It is further noted that if one desires to deliver a diagnostic agent to the above-mentioned parts of the brain, then the diagnostic agent is derivatized accordingly, i.e., eicosapentaenoic acid when glial delivery is required and docosahexaenoic acid when cortical, cholinergic and adrenergic neuron delivery is required. In the latter instance, a targeting/internalization moiety might also be included in the complex through direct attachment to the agent.

10 The present invention further describes a transport vector comprising the above site-specific moiety covalently bonded to a cationic poly-L-lysine carrier having a molecular weight of 1,000 to 50,000 and further bonded (directly or indirectly) to one or more polyunsaturated fatty acid residues. Alternatively, the cationic polyamino acid carrier moiety can be bonded to a phospholipid or diglyceride. The transport
15 vector which contains the site-specific complex and the carrier can contain 1 to 5 molecules of an active agent, (such as an enzyme), or 1 to 5 site-specific complex units (molecules), by random coupling to pendant epsilon-amino groups of the carrier. Other coupling methods are also within the scope of the invention.

20 By controlling the molecular weight of the poly-L-lysine carrier, it is possible to regulate the penetration of the whole macromolecular vehicle (cationic at physiological pH) through the BBB in vivo, and to predict, design and fabricate the adequate drug delivery system with high performance in vivo. When site-specific complexes contain cationic carriers, intravenous administration into mammals is preferred.

25 The site-specific complex of the present invention which includes the active agent and cationic carrier will further contain one or more DHA or EPA moieties so that the complexes may reach their sites of action more effectively. The number of DHA or EPA moieties in such complexes may vary from 1 to 20 moieties.

30 The instant invention is further inclusive of methods for the site directed delivery by means of the above described transport vector of drugs to treat diseases of the glial tissue of a mammalian brain by intravenous administration and penetration

-11-

through the blood-brain barrier (of an afflicted mammal) of a therapeutically effective amount of a biologically active agent also called drug, that has been derivatized with alpha-linolenic acid. When the target tissue is neuronal in nature, the omega-3 fatty acid used for derivatization is DHA.

5 In the following example, provided for illustrative purposes when intravenous administration is thought, three different groups are attached to the main polymeric carrier-chain comprising a biodegradable homopolymer of poly-L-lysine.

1. One or more molecules of the active agent (which is the entity that initiates the therapeutic or physiological response). These become active only after being
10 cleaved from the vehicle. The attachment of the agent can be to the amino terminal of the main chain of the poly-L-lysine carrier or through epsilon-amino groups of L-lysine components of the polymeric carrier (e.g., a side spacer) to the main polymer chain. Agent derivatization with omega-3 polyunsaturated fatty acids, or possibly polyethylene glycol(PEG) and omega-3s, is preferred because the peptide link between
15 the agent and cationic, polymeric carrier is readily hydrolyzed intracellularly by lysosomal enzymes. Besides being directive to the glia (ALA or EPA), or neurons (DHA) this derivatization, in association with PEG, is protective against said hydrolysis. This last process can be optimized by using pH- sensitive spacer molecules between the agent and the carrier. In this example, the agent is an enzyme
20 or a protein, in a protected form, with low antigenicity following administration to mammals..

2. A targeting and/or internalizing moiety can be attached to poly-L-lysine through an epsilon-amino group of L-lysine. This moiety is a specific transport moiety to neuronal brain tissue or cells. Examples of targeting moieties are pH-
25 sensitive groups or receptor-active components, such as antibodies or neurotoxin fragments compatible with a receptor on a specific cell surface, (e.g., tetanus toxin fragment-C (TTC) for cerebral cortical neuronal cells and a nontoxic alpha-bungarotoxin (ABT) fragment for nicotinic acetylcholine receptor of hippocampal neurons) or nerve growth factor (NGF) for cholinergic neurons in general and the
30 neurons of the basal ganglia of Meynart, in particular.

3. One or more hydrophobic groups comprising omega-3 fatty acids or their derivatives can also be attached to the macromolecular carrier by covalent bonds to enhance the lipophilic character of the polar agent transport vehicle. Increased lipophilicity aids incorporation of the complex into the lipid matrix of the microvascular endothelial membrane constituting the blood brain barrier.

The instant invention further comprises site-directed complexes, wherein the lipophilic moiety derived from omega-3 fatty acids or their derivatives is attached to the biologically active agent, such as enzymes, through electrostatic forces. Also, site-specific complexes, wherein said complex molecules are held together through van der Waals forces are within the scope of this invention. Such complexes might have the advantage over the covalently bound conjugates of facilitating the bioavailability of the agent transported into a specific area of the brain without requiring a chemical reaction to achieve the release of the agent from the conjugate.

The resultant site-specific complex interacts with water to form self-assembling structures in colloidal dispersions. Alternatively, the site-specific complex is mixed with lipids, such as phosphatidyl choline and cholesterol, in aqueous systems, to form liposomes by conventional methods. The pharmaceutical preparations are administered in vivo where the said complex is attracted to the specific cell membrane and its uptake to the brain is enhanced. Therefore, the colloidal particles (e.g., micelles, vesicles or liposomes) are specifically targeting a cell surface determinant and subsequently internalized through endocytosis or pinocytosis.

Further, this invention comprises a method for facilitating the transport of an agent into brain cells with said stable colloidal aqueous dispersion of site-specific complexes of the agent. Although the mechanism of the transport is not yet clear, it probably involves the binding of the said complex with brain cells via the excess positive charges on the complex. The lipophilic group of the site-specific complex is a hydrophobic moiety which allows insertion of the complex into the cell membrane or into liposomal bilayers. The cell surface-bound complex is internalized, and the agent is released into the cell.

Enzymes and proteins which are defective in lysosomal storage diseases are listed in Table 2, (entitled "Lysosomal Storage Diseases"). The site specific

-13-

complexes of listed active enzymes and proteins are intended to be utilized in the therapy of diseases mentioned in Table 2, and they are within the scope of the invention. In the case of Gaucher's disease the enzyme is human glucocerebrosidase (or its recombinant form), or modifications thereof, such as alglucerase or ceredase and cerezyme.

The presence of a fusion protein (e.g., viral fusion proteins, influenza hemagglutinin) covalently coupled with the lipophilic moiety through the polylysine carrier or as a targeting moiety of the agent complex in delivery system enhances endocytosis of colloidal particles which are ultimately broken down in the lysosomes.

The fusion proteins (e.g., viral envelope glycoprotein of Sendai virus, influenza virus or herpes Simplex virus) have been exploited for cytoplasmic delivery of macromolecules which are membrane impermeable [Bentz J. et al, "Liposomes, membranes fusion, and cytoplasmic delivery", Chapter 20, in "The structure of biological membranes", Edited by Yeagle Ph., CRC Press, Boca Raton, 1992, pp. 915-947; Hoekstra, D. et al, "Cell biology of entry and exit of enveloped viruses", Chapter 21, in "The structure of biological membranes", Edited by Yeagle Ph., CRC Press, Boca Raton, 1992, pp. 949-996].

Any of the included complexes of this invention can be administered intrathecally, directly infused into the brain or administered intravenously.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows a model of a lipophilic polylysine as an amphipathic conjugate or complex; (a): shows a lipophilic poly-L-lysine conjugate through a peptidic bond between alpha-amino terminal of the main polypeptide chain and free carboxylic group of diacyl phosphatidyl serine (DAPS). Also lyso-phosphatidyl serine may be used.

(b): shows another derivative of poly-L-lysine and DAPS, covalently bound through a peptidic bond between the amino group of DAPS and the carboxyl terminal of the main polymeric chain (shown in the same Figure 1 (b), by dotted line) or through a monoester bond of the main chain and diacyl glycerols. Also, phosphatidyl ethanol amine may be used. R_1 and R_2 represent alkyl chains of fatty acids of omega-3 or omega-6 series.

FIG. 2 is a schematic representation of a lipophilic site-specific biomolecular complex or a targeting vehicle for pharmacologic agents designed by covalent linkage of a fatty acid or a phospholipid molecule (noted as lipophilic moiety: LP) to the agent (A) through a poly-L-lysine (PLL, wherein subscript N has values between zero and 50, preferably 11 lysyls). Optionally, said complex is further coupled with another segment of poly-L-lysine (PLL, wherein subscript M comprises values between zero and 50, preferably 11 lysyls) derivatized with a targeting moiety (T) for selective agent delivery to the brain.

FIG. 3 is a schematic representation of a site-specific biomolecular complex or a targeting vehicle for pharmacologic agents constructed by covalent linkage of a fatty acid or phospholipid molecule (represented by lipophilic moiety) to the agent (A) through a poly-L-lysine (PPL, wherein subscript N is between zero and 11), optionally coupled with another segment of poly-L-lysine (PPL, wherein subscript M is an integer having values between zero and 11) derivatized with a targeting moiety for selective drug delivery to the brain.

FIG. 4 is a schematic representation of a site-specific biomolecular complex or a targeting vehicle of an agent, wherein targeting moiety (b) is coupled with the terminal of the main polymeric chain, and the agent is coupled with epsilon-amino group of the side lysine chain, R_1 and R_2 the same or different, represent alkyl chains (a) saturated in sn1 position of glycerol moiety and poly-unsaturated in sn2 position, suitably of 18 to 46 carbon atoms with 3 to 6 or more double bonds, preferable docosahexaenoyl residue: R_1 and R_2 : $\text{CH}_3\text{-CH}_2\text{-(CH=CH-CH}_2\text{)}_6\text{-CH}_2\text{-}$. Phosphatidyl serine is coupled to main polymeric chain through its -COOH group.

FIG. 5 is a schematic representation of a site-specific biomolecular complex or a targeting vehicle of the agent, wherein targeting moiety (b) is coupled with the terminal of the main polymeric chain, and the agent is coupled with epsilon-amino group of the side lysine chain, R_1 and R_2 the same or different, represent alkyl chains (a) saturated in sn1 position of glycerol moiety and poly-unsaturated in sn2 position, suitably of 18 to 46 carbon atoms with 3 to 6 or more double bonds, preferable docosahexaenoyl residue: R_1 and R_2 : $\text{CH}_3\text{-CH}_2\text{-(CH=CH-CH}_2\text{)}_6\text{-CH}_2\text{-}$. Phosphatidyl serine is coupled to main polymeric chain through its -NH₂ group.

-15-

FIG. 6 is a schematic representation of a site-specific biomolecular complex or a targeting vehicle of the agent, wherein a targeting moiety and the agent are coupled with epsilon-amino group(s) of the side lysine chain, R_1 and R_2 the same or different, represent alkyl chains (a) saturated in sn1 position of glycerol moiety and poly-unsaturated in sn2 position, suitably of 18 to 46 carbon atoms with 3 to 6 or more double bonds, preferable docosahexaenoyl residue: R_1 and R_2 : $\text{CH}_3\text{-CH}_2\text{-(CH=CH-CH}_2\text{)}_6\text{-CH}_2\text{-}$. Phosphatidyl serine is coupled to main polymeric chain through its -NH_2 group. Subscript N has values between 1 and 25.

FIG. 7 shows a site-specific biomolecular complex or a targeting vehicle of a biologically active agent, wherein the agent is derivatized with a lipophilic carrier. Lipophilic moiety is represented by a fatty acid of omega-3 and omega-6 series (a), with hydrocarbon chain of 18 to 46 carbon atoms, preferable poly-unsaturated of 22 carbon atoms with 6 double bonds, i.e., docosahexaenoyl residue: $\text{CH}_3\text{-CH}_2\text{-(CH=CH-CH}_2\text{)}_6\text{-(CH}_2\text{)-}$. Targeting moiety is shown in the same figure (b).

DESCRIPTION OF THE PREFERRED SPECIFIC EMBODIMENTS

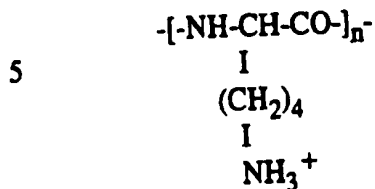
The lipophilic functional poly-unsaturated molecules which form part of the site-specific complex are preferably selected from the group consisting of fatty acids of the omega-3 series or lipid derivatives thereof. Other examples of lipophilic molecules are fatty acids, diacyl glycerols, diacyl phospholipids, lyso-phospholipids, cholesterol and other steroids, bearing poly-unsaturated hydrocarbon groups of 18 to 46 carbon atoms.

Preferred bio-polymer carriers are poly (alpha)-amino acids (e.g., PLL, poly L-arginine:PLA, poly L-ornithine:PLO), human serum albumin, aminodextran, casein, etc. These carriers are biodegradable, biocompatible and potentially excellent candidates for drug delivery systems.

The present invention is illustrated by the use of the following preferred specific embodiments, as non-limiting examples.

MACROMOLECULAR CARRIER

The macromolecular carrier is a polypeptide carrier exemplified by poly-L-lysine which is represented by the following structural formula:



wherein subscript n denotes the number of L-lysine in the carrier.

10 Another variant of the poly-lysine carrier that is within this invention contains the lysines, either D- or L-configuration, linked alternatively by typical peptide bonds to each other.

 Another polypeptide that is within the scope of this invention is a C-terminal and N-terminal arginine or the masked or blocked arginine. Preferred N-terminal
15 amino acid groups are arginine, acetyl arginine, lysine or acyl lysine, where these terminal amino acids are of either D- or L-configuration. The amino acids that constitute the core sequence of the polypeptide of this invention are preferably in the L-isomer form. Characteristic features and structural/colloidal behavior of the polypeptides of this invention are essential to allow the proper polymer
20 configuration/conformation to effect an increase of permeability of BBB when they are attached to a drug administered to the host animal. The host can be humans, domestic animals (e.g., dog, cat, horse, etc.), and animals for experimental purposes (e.g., rabbits, rats and mice) or any animal which possesses a central nervous system (i.e., a brain).

25 If the D-isomer of lysine is optionally added for one or more times in the linear core sequence of lysines, particularly in C-terminal region, the penetration of the drug-conjugates through the BBB might be altered, even diminished. In the preferred macromolecular embodiment of this invention the polylysine should be derived from the L-isomer.

30 Known preparative methods can be used for synthesis of PLL, with desired average molecular weight: MW. The hydrobromide salts of PLL of varying MW's are also commercially available, for example, hydrobromide salts of PLL of MW

-17-

3300-4000 (i.e., PLL with about 26 lysyls chain length) and of MW 180,000 - 260,000 (i.e., 1200-1700 lysyls in poly-lysine chain length), etc. In general, polypeptides with less than 20 amino acids are prepared by known solid phase synthesis.

5 Thus, a preferred embodiment of this invention is poly-L-lysine, noted also as PLL or poly-Lys, with n comprised between 6 and 25, sometimes at least 50 lysine molecules as a constituent part of a site-specific complex. For an understanding of the structural and functional role of poly-Lys as a macromolecular carrier we developed an artificial model system (i.e., a drug vehicle) using lipophilic poly-Lys
10 carrier for drug delivery systems.

 This invention also pertains to lipophilic poly-Lys conjugates or complexes wherein a wide variety of lipophilic compounds (e.g., highly unsaturated fatty acids or derivatives thereof) are covalently bound to linear (Figure 1) or branched poly-Lys. Examples of highly unsaturated fatty acid derivatives such as phospholipids are, but
15 not limited to those, listed in Table 1.

TABLE 1
PREFERRED SELECTED PHOSPHOLIPIDS
TO BE USED LIOPHILIC MOIETY

- | | | |
|----|----|---|
| 5 | 1. | (rac)-1-O-octadecanoyl, 2-O-docosahexaenoyl-glycero-3-phospho-serine |
| | 2. | (rac)-1-O-octadecyl, 2-O-docosahexaenoyl-glycero-3-phospho-serine |
| | 3. | (rac)-1-O-hexadecanoyl, 2-O-docosahexaenoyl-glycero-3-phospho-serine |
| 10 | 4. | (rac)-1-O-hexadecyl, 2-O-docosahexaenoyl-glycero-3-phospho-serine |
| | 5. | (rac)-1-O-octadecanoyl, 2-O-eicosapentaenoyl-glycero-3-phospho-serine |
| 15 | 6. | (rac)-1-O-octadecyl, 2-O-eicosapentaenoyl-glycero-3-phospho-serine |
| | 7. | (rac)-1-O-hexadecanoyl, 2-O-eicosapentaenoyl-glycero-3-phospho-serine |
| | 8. | (rac)-1-O-hexadecyl, 2-O-eicosapentaenoyl-glycero-3-phospho-serine |
| 20 | 9. | (rac)-1,2-O-di-docosahexaenoyl-glycero-3-phospho-serine |

-19-

10. (rac)-1, 2-O-di-docosahexaenoyl-glycero-3-phospho-ethanolamine
11. (rac)-1, 2-O-di-docosahexaenoyl-glycero-3-phospho-ethanol mono methyl amine
- 5 12. (rac)-1-O-hexadecyl, 2-O-docosahexaenoyl-glycero-3-phospho-carnitine
13. (rac)-1 -O- docosahexaenyl, 2-O-docosahexaenoyl-glycero-3-phospho-carnitine
14. (rac)-1, 2-O-di-docosahexaenyl-glycero-3-phospho-carnitine
- 10 15. (rac)-1-O-hexadecyl, 2-O-docosahexaenoyl-3-[2-(2-mono methylammonio acetyl amino)ethyl]phosphate
16. (rac)-1-O-octadecanoyl, 2-O-eicosapentaenoyl-glycero-3-phospho-ethanol amine acylated derivative
17. (rac)-1-O-eicosapentaenyl, 2-O-eicosapentaenoyl-glycero-3-phospho-serine
- 15 18. (rac)-1-O-eicosapentaenyl, 2-O-eicosapentaenoyl-glycero-3-phospho-ethanol amine acylated derivative.
19. (rac)-1, 2-O-di-eicosapentaenoyl-glycero-3-phospho-serine
20. (rac)-1, 2-O-di-dihomo-gamma-linolenoyl-glycero-3-phospho-serine
- 20

-20-

- 21 (rac)-1-0-dihomo-gamma-linolenoyl, 2-0-docosahexaenoyl-glycero-3-phospho-serine
- 22. (rac)-1-0-dihomo-gamma-linolenoyl, 2-0-eicosapentaenoyl-glycero-3-phospho-serine

The acyl derivatives of phosphatidyl serine (DAPS) are not critical, but in general (see, Table 1) will contain at least 16 carbon atoms. Preferably the fatty acyls R_1 and R_2 contain from 16 to 24 carbon atoms exclusive of additional side chains or functional groups. The fatty acyls may be saturated (in sn1-position of glycerol backbone) and poly-unsaturated of omega-3 or omega-6 essential fatty acid series (in sn2-position) or both poly-unsaturated with at least 18 carbon atoms. Suitable fatty acids include palmitic (PA), stearic (STA), linoleic (LO), linolenic (LA), arachidonic (AA), di-homo gamma linolenic (DGLA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids.

Other lipophilic poly-lysine conjugates can be formed through one or more peptidic bonds between amino group(s) of the carrier and carboxylic group(s) of a poly-unsaturated fatty acid(s) of omega-3 or omega-6 series, preferably DHA, EPA and DGLA.

Another alternative to design lipophilic poly-Lys is by using branched poly-Lys, for example with a single lysine like a free block.

The lipophilic-PLL carrier of pharmaceutical compositions of this invention can also contain neuropharmaceutical agents which have a prophylactic and/or therapeutic effect on neurological disorders. For example, neurological disorders include: malignant brain tumors, autoimmune deficiency syndrome (AIDS), Parkinson's disease, neurodegenerative disease, Alzheimer's disease, multiple sclerosis, migraine, pain or a seizure disorder, epilepsy, depression and trauma, neuronal storage disease, and other severe deterioration of the CNS.

This invention also relates to the design of novel drug-vehicles or drug-shuttles with increased lipophilicity and increased penetration into biological membranes having facilitated transport throughout the body. The invention is also particularly useful for delivering to neuronal sites pharmaceutically active agents such as anti-neoplastic agents, anti-microbial agents, anti-parasitic agents, adrenergic agents and catecholaminergic agents, anti-convulsants, nucleotide analogues, anti-trauma agents, enzymes and proteins used to prevent or treat neurological disorders.

In the model of targeting lipophilic complexes, (presented in Figure 2), both the therapeutic agent and targeting moiety are attached at the terminals of the main

-22-

linear chain of the poly-peptide. Alternative variants of the derivatization approach presented above (Figure 2) in which the agent is coupled with epsilon-amino group of side lysyl chain (see Figures 3-7) or to branched poly-lysine are also preferred features of this invention.

5 From the description of novel concept of targeting lipophilic complexes or conjugates, and from the above examples, one skilled in the art can easily ascertain the characteristic essential features of this invention, without departing from invention objectives, and can make various changes in the invention complex model to adapt it to various usages and conditions.

10 The molecular conjugates of this invention are prepared conventionally by known methods in the art, as shown in the following examples.

PREPARATION OF ENZYMES COVALENTLY COUPLED WITH POLY-UNSATURATED FATTY ACIDS

GENERAL DESCRIPTION

15 The molecular conjugates of an agent (A), such as a biologically active enzyme (which can be noted E), with poly-unsaturated fatty acids, noted PUFA, may be represented by the general formula:

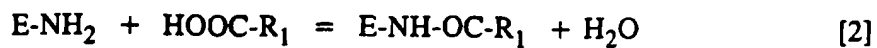


[1]

20 wherein r is an integer from 1 to 20; and PUFA is chosen from a group consisting of omega-3 essential PUFA, more preferably alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and most preferably docosahexaenoic acid (DHA) and represented by the formula R_1-COOH .

25 These conjugates of formula [1] may be prepared by condensation of R_1-COOH with the appropriate amine group(s) on the enzyme $E-NH_2$. Many methods for the formation of amides from acids and amines are known in the art. Among them, the use of carbodiimides is particularly recommended for compounds of the above general formula. The reaction for conjugation of E with PUFA is illustrated by:

-23-



The starting materials are readily available, or their synthesis and purification are well known in the art.

5 The enzyme derivatives of the present invention are particularly useful for treating lysosomal storage diseases which cause severe neurodegeneration and are manifested by enzyme deficiencies. The specific enzymes which can be derivatized are, but not limited to those, listed in Table 2 [Neufeld, Annu. Rev. Biochem., 60:257-80 (1991)].

TABLE 2
LYSOSOMAL STORAGE DISEASES

	<u>Disorder</u>	<u>Deficient Enzyme</u>
	1. <u>Sphingolipid degradation</u>	
5	Farber's disease	ceramidase
	Fabry's disease	alpha-galactosidase
	Gaucher's disease	glucocerebrosidase
	G _{M1} gangliosidosis	beta-galactosidase
	G _{M2} gangliosidosis:	
10	Tay-Sachs disease	beta-hexosaminidase A
	Sandhoff disease	beta-hexosaminidase A & B
	Activator deficiency	G _{M2} activator
	Krabbe disease	galactosylceramidase
	Metachromatic leukodystrophy	enzyme-deficient form arylsulfatase A
15		activator-deficient form sulfatide
		activator/saposin
	Mucopolipidosis IV	ganglioside sialidase
	Multiple sulfatase deficiency	all sulfatases
	Niemann-Pick disease	sphingomyelinase
20	Schindler disease	alpha-N-acetylgalactosaminidase
	2. <u>Glycoprotein degradation</u>	
	Aspartylglycosaminuria	aspartylglycosaminidase
	Fucosidosis	alpha-L-fucosidase
	Galactosialidosis	protective protein/cathepsin, beta-galactosidase and sialidase
25	alpha-Mannosidosis	alpha-mannosidase

-25-

beta-Mannosidosis

beta-mannosidase

Sialidosis

sialidase

3. Glycosaminoglycan degradation

	Hunter syndrome	iduronate sulfatase
5	Hurler and Scheie syndromes	alpha-L-iduronidase
	Maroteaux-Lamy syndrome	GalNAc 4-sulfatase/arylsulfatase B
	Morquio syndrome	
	A subtype	Gal 6-sulfatase
	B subtype	beta-galactosidase
10	Sanfilippo syndrome	
	A-subtype	heparan N-sulfatase
	B-subtype	alpha--N-acetylglucosaminidase
	C-subtype	AcetylCoA: glucosamine N- acetyltransferase
15	D-subtype	GlcNAc-6-sulfatase
	Sly syndrome	beta-glucuronidase

4. Other single enzyme deficiency disorders

	Pompe disease	
	(glycogenosis II)	alpha-glucosidase
20	Wolman disease	acid lipase

5. Disorders of lysosomal enzyme biosynthesis

	I-cell disease and pseudoHurler polydystrophy	6-phospho-N-acetylglucosamine transferase
--	--	--

-26-

6. Disorders of lysosomal membrane transport

Cystinosis

cystine transport

Sialic storage and

Salla disease

sialic acid transport

-27-

The coupling of PUFA with reactive amino acids on enzyme, preferably lysine through its epsilon amino group or with the N-terminal amino group of the enzyme, may be achieved through amide bond as shown in reaction [2].

The reaction product,



wherein r is an integer from 1 to 20 which shows the varying numbers of lipophilic residues attached to the enzyme macromolecule and is controlled primarily by the ratio of PUFA to lysyls of enzyme chain length and secondarily by the duration of the coupling reaction. The composition and structure may be determined for any
10 condensation product by the known method in the art (Lowry et al., J. Biol. Chem., 193, 265-275 (1951)).

The conditions for reaction [2] are conventional conditions for the formation of an amide linkage, such as by activating the fatty acid ($HOOC-R_1$) with a suitable activator under non-aqueous conditions, for example dicyclohexylcarbodiimide, and
15 subsequent reaction with the appropriate amine group(s) on enzyme. During reaction, pH may be controlled, if necessary, by acid or base addition either manually or automatically.

After the conjugation reaction is complete, excess reagent and unwanted reaction products may be removed by suitable techniques known in the art, including
20 chromatographic procedures, for example gel filtration, dialysis and diafiltration, etc. Such conjugations are further described by examples 1 to 3.

EXAMPLE 1

In a practical example, an enzyme is dissolved in an aqueous buffer preferably at a moderately alkaline pH 7-9.5 (e.g., phosphate buffered saline: PBS). At a
25 temperature in the range of 0 to 40°C, the solution of enzyme is treated with 0.6 equivalents of PUFA dissolved in non-aqueous medium in the presence of 1 equivalent of suitable carbodiimide, preferably dicyclohexylcarbodiimide, under the argon and suitable anti-oxidant compounds to protect from damage the PUFA during

the conjugation reaction. The reaction is permitted to proceed for about 17 hours at ambient temperature. Then the reaction mixture is passed through Sephadex columns. The reaction product provides around 2 PUFA residues per enzyme, (see formula [3]).

5 In a similar manner, various molecular conjugates with different stoichiometric ratios of PUFA per enzyme molecule could be prepared.

Carbodiimide coupling is a well-known procedure outlined in the following literature references: Sheehan, J. C. and Hess, G.P., "New method of forming peptide bonds", J. Am. Chem. Soc., 77, 1067 (1955); Halloran, M.J. and Parker, C.
10 W., "The preparation of nucleotide-protein conjugates: Carbodiimides as coupling agents", J. Immunology, 96, 373 (1966); Kurzer, F., and DouraghiZadeh, K., "Advances in the chemistry of carbodiimide", Chem. Rev., 67, 107 (1967); Carraway, K.L. and Koshland, Jr., D. E., "Modification of proteins by carbodiimide reaction", Methods in Enzymology, Vol. 25B, p. 616 (1972).

15 The molecular conjugates of enzyme and PUFA within this invention are also prepared conventionally as illustrated in examples 2 and 3.

EXAMPLE 2

Method for preparation of enzyme covalently coupled with PUFA using the conjugation reaction between enzyme and N-hydroxysuccinimide ester of PUFA in
20 phosphate buffered saline (PBS) at pH 6-9.5, containing deoxycholate. For example, 3 mg of purified enzyme is added to 50 microgrammes of N-hydroxysuccinimide ester of docosahexaenoic acid in PBS containing 2-3% deoxycholate. The mixture is incubated at 37°C for 9 to 15 hrs and then chromatographed on a Sephadex G-75 column (1.3 x 45 cm) in PBS containing 0.15 % deoxycholate to remove excess of
25 PUFA. Such reactions are described in the prior art: Huang et al., J. Biol. Chem., 255(17), 8015-8018 (1980), Lapidot et al., J. Lipid Res., 8, 142-145 (1967).

-29-

EXAMPLE 3

Conjugation of the enzyme with fatty acids of omega-3 series (such as, DHA) can be carried out according to known procedures for reacting amino groups on enzyme with carboxyl group of poly-unsaturated fatty acids (as shown in example 1) or with their acyl chlorides or anhydrides. For example, enzyme is dissolved in bicarbonate buffer (BB, of pH 9); then PUFA-anhydride, dissolved in anhydrous dimethyl sulfoxide (DMSO), is added immediately to the enzyme solution. Enzyme-PUFA complexes of formula [1] are prepared. The stoichiometric ratio of PUFA/Enzyme could be varied as justified already. The reaction is allowed to proceed for 2 hours at 25 °C. Then, the reactants were passed through G-25 Sephadex columns (using the procedure of U.S. Pat. No. 5,230,883). In a similar manner, PUFA-polymer conjugates at desired stoichiometric ratio, i.e., at a particular value of r in formula [1] can be prepared by routine experiments as stated above.

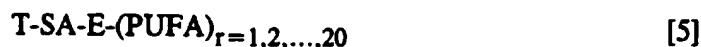
Various changes and permutations of the constituents of the transport vector of an enzyme, (e.g., of the lipophilic enzyme conjugates), can be further derivatized through several examples:

The reaction product,



of an enzyme derivatized with PUFA and chemically linked to a protective moiety to decrease the enzyme antigenicity, e.g., human serum albumin (SA) or polyethylene glycol (PEG), is a non-immunogenic, protected-enzyme-PUFA conjugate.

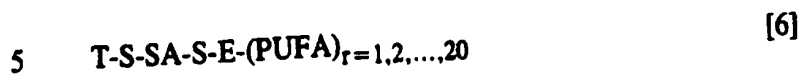
The reaction product,



is a targeted non-immunogenic, protected enzyme-PUFA vehicle, wherein T stands for the targeting moiety for specific delivery of the resultant conjugates to selective cellular surface receptors. T is selected from the group consisting of tetanus toxin fragment C (TTC), nerve growth factor (NGF), alpha-bungarotoxin (ABT), cell-

surface directed antibodies (such as immunoglobulin G, antibody against human pancreatic tumor cells, and antibody against hepatocytes) and insulin as an example of a serum hormone used as targeting moiety, depending on the desired site of delivery.

The reaction product;



- is an acid-sensitive targeting non-immunogenic, protected enzyme-PUFA conjugate, i.e., a large, complex vehicle targeting non-immunogenic and protected enzyme-PUFA conjugate, designed for a cytoplasmic delivery of the enzyme after parenteral administration (in blood circulation). S is a spacer molecule which is pH sensitive.
- 10 Accordingly, the resultant molecular conjugate [6] is designed for specific cytoplasmic delivery of the enzyme.

Various reaction products involved in the synthesis of the resultant enzyme conjugates of [6] are achieved using sequential step reaction procedures (see, conjugates of [4] and [5]).

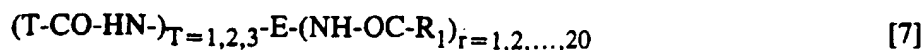
15 PREPARATION OF ENZYMES (OR PROTEINS) COVALENTLY COUPLED WITH POLY-UNSATURATED FATTY ACIDS AND TARGETING MOIETIES AND/OR CARRIERS

Some of the various reaction products involved in the synthesis of molecular conjugates using step reaction procedures are shown below.

- 20 As targeting moieties: T, tetanus toxin fragment C :TTC or poly L-lysine:PLL are used as examples, but not limited to them. PLL could be used as a targeting moiety in terms of effecting cellular uptake of its molecular conjugates to an agent (enzyme) and also as a cationic transport carrier under the physiological conditions.

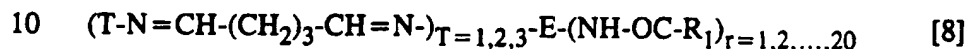
- 25 The molecular conjugates of a biologically active enzyme (or a therapeutic agent:A), which is noted as E, with poly-unsaturated fatty acids, noted as PUFA and symbolized by $R_1\text{-COOH}$, E are also covalently coupled with targeting moieties T, and may be represented by the following formula:

-31-

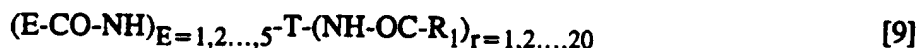


wherein subscript T is an integer with varying values from 1 to 3 and r has the known meaning. Briefly, the molecular conjugates of [7], can be written as T-E-PUFA conjugates, where covalent linkages between different parts of the transport vehicle
5 are omitted, for the sake of simplicity.

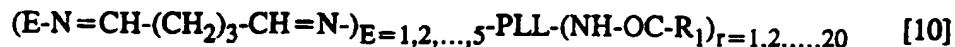
The resulting T-E-PUFA conjugates [7] are synthesized by carbodiimide coupling procedure. Also, the T-E-PUFA conjugates can be achieved by a complex method, using glutaraldehyde coupling procedure and carbodiimide coupling procedure, and the resulting conjugates are given by [8]:



Permutations of the constituents on the transport vector [7] give new E-T-PUFA conjugates of [9]:

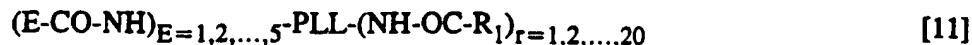


The E-T-PUFA conjugates are synthesized by carbodiimide coupling
15 procedure. Also, the E-T-PUFA conjugates [10]:



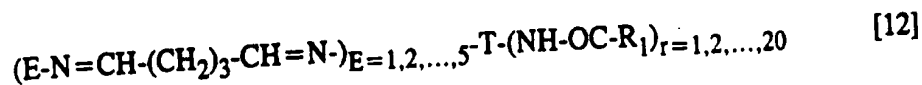
are prepared in two steps using both glutaraldehyde coupling procedure and carbodiimide coupling procedure.

Similar various molecular conjugates of an enzyme, chemically coupled with
20 PLL carrier and with PUFA are given by [11]:



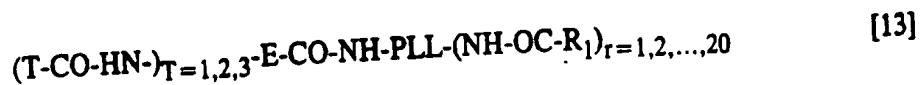
wherein subscript E is an integer with values from 1 to 5 and shows the plurality of the agent (noted A or E, when is particularized by an enzyme) on the transport vectors. The E-PLL-PUFA conjugates represented by [11] are achieved by carbodiimide coupling procedure. Also, similar E-PLL-PUFA conjugates given by

5 [12]:

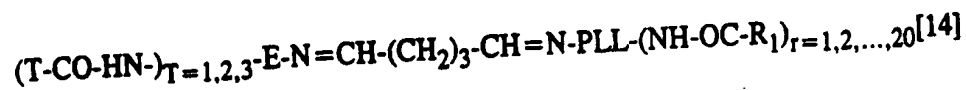


are prepared in two steps using glutaraldehyde coupling procedure and carbodiimide coupling procedure.

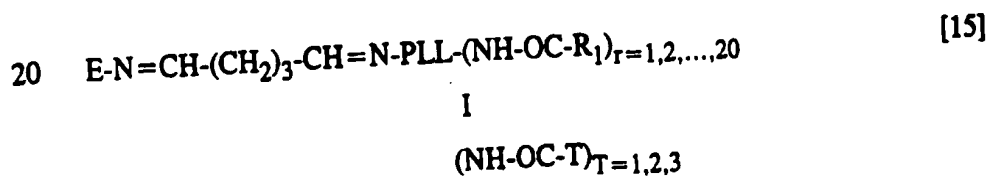
10 Molecular conjugates of an agent (i.e., enzyme) with PLL carriers, targeting moiety and PUFA are given by [13]:



The resulting T-E-PLL-PUFA molecular conjugates are prepared in three steps using carbodiimide coupling. Also, a mixed method using a combination of glutaraldehyde coupling procedure and carbodiimide coupling procedure could be
15 achieved and the resulting T-E-PLL-PUFA molecular conjugates are illustrated by [14]:



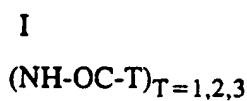
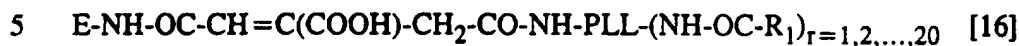
Various changes and permutations of the constituents on the transport vectors [14] can be realized and the resulting conjugates are illustrated by [15]:



The resulting conjugates of [15] could be symbolized by E-PLL(T)-PUFA.

-33-

Further, a pH-sensitive spacer molecule (noted as S) can be inserted between agent (such an enzyme) and PLL carrier or between other parts of the transport vehicles, depending on the desired properties of the resulting final conjugate. Examples are illustrated by [16] and [17]:



The resulting conjugates given by [16] and [17] can be symbolized by E-S-PLL(T)-PUFA.

Accordingly, conjugation can be achieved by chemical reactions, using step reaction procedures, well known in the art, employing carbodiimide catalyst, or
15 combinations of glutaraldehyde coupling and carbodiimide coupling modes.

EXAMPLES OF STEP REACTION PROCEDURES

The molecular conjugates [7] of the following formula $(\text{T-CO-HN-})_T\text{-E-(NH-OC-R}_1\text{)}_r$, written also in T-E-PUFA form, wherein subscripts T and r have their known meaning, are prepared conventionally in two steps.

20 In step I, there is described and obtained a conjugated product of enzyme with PUFA, i.e., $\text{E-(NH-OC-R}_1\text{)}_r$, as shown in examples 1 to 3.

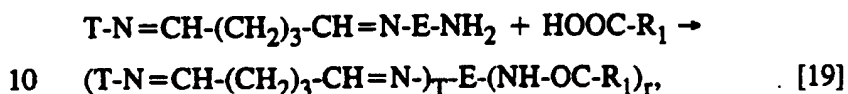
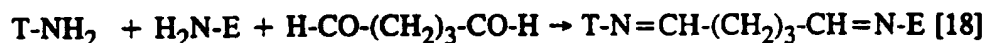
In step II, the conjugated product of step I, $\text{E-(NH-OC-R}_1\text{)}_r$, is covalently coupled with a specific target moiety. This reaction is carried out according to known procedures for reacting amino groups on enzyme with carboxyl groups of the
25 targeting moiety to provide linkage between them, for example using carbodiimide coupling as in step I (see, example 1).

-34-

Other modes of conjugation can be used, depending on the structure and properties of the individual molecules employed to build the vehicle. For example, conjugation can be made by using bifunctional protein modifying reagents, such as glutaraldehyde, resulting in the following conjugated product of [8], (T-N=CH-

5 (CH₂)₃-CH=N-)_T-E-(NH-OC-R₁)_r, represented further by formula T-E-PUFA.

The conjugated T-E-PUFA product can also be achieved by the following reactions:



The conjugation reaction [18] may be achieved using a variety of bifunctional protein modifying reagents. Examples of such reagents include: N-succinimidyl-3-(2-pyridyldithio)propionate, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde used in reaction [18].

15 Glutaraldehyde coupling is described in the prior art: Avrameas, S. and Ternynck, T., "Peroxidase labelled antibody and Fab conjugates with enhanced intracellular penetration", *Immunochemistry*, 8, 1175-1179 (1971); Gonatas, N. K., Kim, S. U., Stieber, A. and Avrameas, S., "Horseradish peroxidase-lectin conjugates", *J. Cell Biol.*, 73, 1-13 (1977). Undoubtedly, other modes of

20 conjugation can be used, depending on the structure and properties of the individual molecules employed to build the vehicle.

The conjugated product of reaction [18] is further derivatized with PUFA, following the procedure of reaction [19].

In a similar manner, a wide variety of molecular conjugates with different

25 stoichiometric ratios of PUFA per enzyme molecule could be prepared.

Alternatively, conjugates of formulae [9] to [17] can be made in the intermediate steps, depending on the enzyme placement in the conjugated individual vehicle molecule.

-35-

As targeting moiety, tetanus toxin fragment C :TTC can be used as an example, but not limited to.

EXAMPLE 4

5 PREPARATION OF LIPOPHILIC POLY-LYSINE BY CONJUGATION OF POLY-LYSINE TO POLY-UNSATURATED FATTY ACIDS

The reactants of example 1 are used, except that 10 mg of the hydrobromide salts of poly-L-lysine: PLL of average molecular weight of 3300-4000 (containing about 25 lysyls in PLL chain length) is substituted for the enzyme. The quantity of PUFA, such as DHA, is calculated for a chosen stoichiometric ratio PUFA/PLL of two. The molecular complex of (DHA)₂-PLL is purified as known in the art.

EXAMPLE 5

15 PREPARATION OF MOLECULAR CONJUGATES OF POLY-LYSINE WITH PHOSPHOLIPIDS AS PHOSPHATIDYL SERINE

The reactants of example 4 are used, except that phosphatidyl serine ((rac)-1-O-octadecanoyl, 2-O-docosahexaenoyl-glycero-3-phospho-serine, named also 1-O-stearoyl, 2-O-docosahexaenoyl-glycero-3-phospho-serine:SDPS) is substituted for the PUFA with the stoichiometric ratios of 2 for SDPS/PLL-macromolecule.

EXAMPLE 6

20 MOLECULAR CONJUGATES OF POLY-LYSINE WITH PHOSPHOLIPIDS

In a similar manner, as in example 5, but using a large variety of other phospholipids (sn1-saturated, sn2-poly-unsaturated, or both chains poly-unsaturated) similar derivatives can be prepared.

EXAMPLE 7

25 CONJUGATION OF ENZYME TO MOLECULAR CONJUGATES OF POLY-LYSINE WITH POLY-UNSATURATED FATTY ACIDS (DOCOSAHEXAENOIC ACID : DHA)

The reactants of example 1 are used, except that molecular conjugate of poly-lysine with DHA (product of example 4) is used to substitute for the PUFA. The

-36-

quantity of said reactants, is calculated for a chosen stoichiometric ratio (see formula [9]) within the desired limits for the hydrophilic hydrophobic balance of the resultant transport vector of enzyme. The final molecular complex of lipophilic enzyme must be present with at least 70% of cationic charges initially available (unmodified PLL)
5 for excellent uptake into cell.

EXAMPLE 8

SYNTHESIS OF PLL-(SDPS)₂ (POLY LYSINE AND DIACYL PHOSPHATIDYL SERINE) CONJUGATES

PLL-(SDPS)₂ conjugates or complexes are synthesized by coupling SDPS to
10 the alpha-amino group and to the carboxyl group of a suitably protected epsilon-amino PLL molecule (e.g., about 12 lysyls in a PLL chain length) using carbodiimide coupling procedure in a similar manner as in example 4.

The reactants of example 4 are used, except that sodium salt of phosphatidyl serine ((rac)-1-O-octadecanoyl, 2-O-docosaheptaenoyl-glycero-3-phospho-serine, named
15 also 1-O-stearoyl, 2-O-docosaheptaenoyl-glycero-3-phospho-serine:SDPS) is substituted for the PUFA with the stoichiometric ratios of 2 for SDPS/PLL-macromolecule and PLL molecule has epsilon-amino groups protected.

Briefly, the coupling reaction entailed adding a 2% solution of SDPS in ethanol in aliquots to a stirred 0.1% solution of the modified (epsilon-amino
20 protected) PLL in 0.01 mol/l carbonate buffer at pH 9, or 0.01 mol/l phosphate buffer at pH 7.5 in the carbodiimide presence, at 0°C. The input molar ratio of SDPS to PLL is two (i.e., 1 SDPS to 6 lysyls), and of buffer to ethanol, 100:1 (v/v). Stirring was continued for 3 hours and the reaction was allowed to proceed overnight (about 14 hrs) at 4°C under the argon and in the presence of antioxidants to protect
25 poly-unsaturated compounds against oxidation. The resulting solution was dialyzed extensively against water or 0.1 mol/l NaCl (pH 7.3) for 3 days. Protecting groups were removed completely from PLL molecular conjugate with HBr in glacial acetic acid. The deprotection was confirmed by ultraviolet spectroscopy at 254 nm.

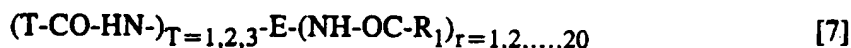
-37-

The SDPS content of the conjugates is determined spectrophotometrically and the analytical data on conjugates show that SDPS is coupled to alpha-amino group and to carboxylic terminal group of PLL molecule.

EXAMPLE 9

5 COVALENT BONDING OF ENZYMES TO PUFA'S AND TO TARGETING MOIETIES

The molecular conjugates of the type [7]:

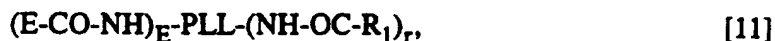


are prepared conventionally in two steps. The molecular conjugate of the first step,
10 enzyme derivatized with PUFA, such as DHA, described in examples 1-3 is covalently coupled, in a second step of conjugation reaction, to a specific targeting moiety, such as tetanus toxin fragment C (TTC). The second conjugation is carried out according to known procedures, for example, reacting amino groups on enzyme with carboxyl groups of targeting moiety (if available) to provide linkage between
15 them.

EXAMPLE 10

PREPARATION OF ENZYME-POLYLYSINE-PUFA CONJUGATES

The molecular conjugates of the type [11]:



20 wherein E is an integer with values from 1 to 5 and PLL is a cationic polymeric-carrier, such as poly-lysine with different molecular weights, are prepared conventionally in two steps. For the sake of simplicity, the value of $E = r = 1$ is chosen.

The molecular conjugate of the first step, PLL derivatized with PUFA, such as
25 DHA, described in example 4, is covalently coupled, in a second step of conjugation reaction, to a specific enzyme. The second conjugation is carried out according to known procedures, for example, reacting amino groups on PLL with carboxyl groups of enzyme (if available) to provide linkage between them.

-38-

Briefly, crystalline enzyme (glucocerebrosidase), poly-L-lysine (of molecular weight of 6700) derivatized with DHA at alpha-amino group, and 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) were dissolved in equal amounts of 10 mg each in 1 ml water. This solution was incubated at room temperature (around 22°C) for 7 hours under argon with occasional shaking, and then loaded onto a Sephadex G-100 chromatographic column which had been previously equilibrated with 0.01 M phosphate buffered saline (PBS), pH 7. After loading, the column was eluted with PBS and fractions containing enzyme coming out of the column at and around the void volume were collected, pooled, concentrated to a volume of 1.0 ml, and then diluted with water to a volume of 10 ml. In order to remove unreacted enzyme, this solution was passed through a DEAE-Sephadex column.

EXAMPLE 11

CONJUGATION OF HORSERADISH PEROXIDASE TO PLL-DHA CONJUGATES

The coupling modes and reactants of previous example 10 were used except that 10 mg of horseradish peroxidase (HRP) was taken for enzyme.

The HRP-PLL-DHA conjugates were used in experiments. The enzymatic activity of the pooled HRP-PLL-DHA conjugates was compared to that of unconjugated HRP using an assay employing dianizidine as an electron acceptor (see, U.S. Pat. No. 4,847,240). It was found that the conjugation decreased the enzymatic activity of HRP by about 40%.

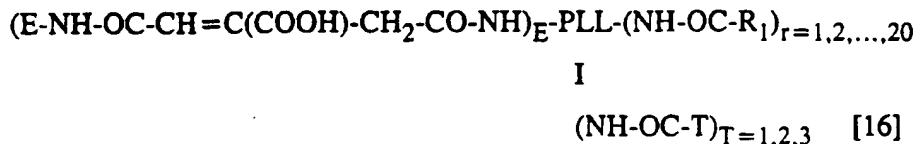
Each elution fraction was also tested for transport into L929 fibroblast cells. The results show that the HRP-PLL-DHA fractions (conjugates) which enter cells most effectively are the first fractions eluted from the Sephadex column, hence the fractions containing the conjugates of largest molecular weight.

-39-

EXAMPLE 12

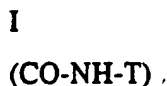
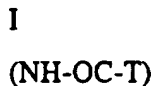
**ACIDITY SENSITIVE MOLECULAR CONJUGATES OF
ENZYME-SPACER-PLL-PUFA
OPTIONALLY COUPLED WITH TARGETING MOIETIES**

5 Molecular conjugates of the general form [16]:



are prepared by linking the enzyme to the PLL carrier through an intermediate
10 molecule, such as maleic anhydride, or copoly (ethylene maleic anhydride), or more
preferable cis-aconitic acid, known as pH-sensitive spacer molecules, illustrated for E
= T = r = 1.

Therefore, a preferred molecular conjugate can be given either by formulae
[20] or [21]:



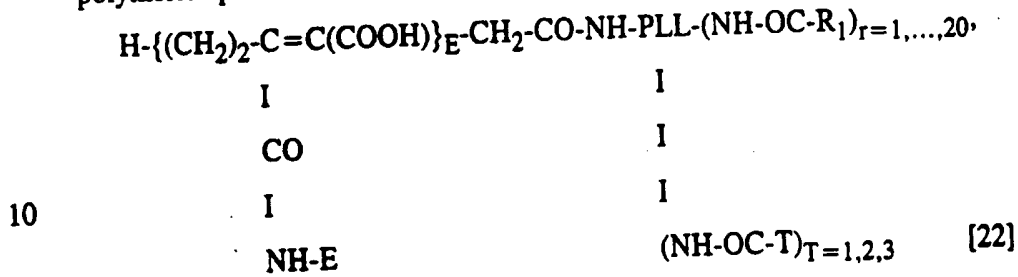
20

Other modes of carrying out this invention can be proposed using spacer
molecules which contain more than one maleic anhydride ring, i.e., poly maleic
anhydride, also commercially available.

The molecular conjugate of example 12 presents the advantage that is pH
25 sensitive and in mild acidic conditions will release easily the enzyme in unmodified
form, which is in a biologically active state. Using these molecular conjugate of

example 12 it is possible to control the intracellular release of the enzyme (e.g., within the lysosome).

Using poly-anhydride, each pair of cis-carboxyl groups formed by hydrolyzing a maleic anhydride is believed to comprise a suitable location for bonding an enzyme molecule. Therefore, numerous enzyme molecules may be bonded to a single polymeric spacer molecule, as shown in formula [22]:



Molecular conjugates of formulae [22] show high efficiency to transport and release numerous enzyme molecules using a single transport vector. The use of a polymeric spacer molecule with poly cis di-carboxylic groups, such as in formula [22], allows for an efficient transport of enzyme (or proteins) in cell lysosomes, and assures a spontaneous release of all enzyme molecules when the resultant conjugate is subjected to acidic conditions.

Various preferred embodiments (e.g., formulae [16], [20]-[22]) within the instant invention can also be achieved by altering the sequence of reactions.

EXAMPLE 13

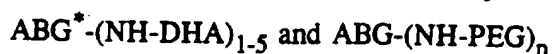
ENZYME REPLACEMENT THERAPY IN TYPE 2 AND 3 GAUCHER DISEASE

The enzyme selected to further exemplify the present invention is the native acid beta-glucosidase (ABG) or glucocerebrosidase, its mannose-terminated derivative alglucerase (mABG) or ceredase, its recombinant acid beta-glucosidase from (rABG) and its recombinant manose-terminated form cerezyme (mrABG). It appears to be five reactive epsilon amine groups of lysines in each form of the enzyme, i.e., in ABG, mABG, rABG, and mrABG. These positions can be partially or fully

-41-

docosaehaenoylated (DHA) or eicosapentaenoylated (EPA). For reasons of similarity between the EPA and DHA structures, we are utilizing DHA in all examples below.

Polyethyleneglycol (PEG) derivatized ABG and its other forms have been reported recently [Martin, B.M., Gladzieka, M., Eliason, W., et al., Amer. J. Hum. Gen., 1992 (51;A307)]. If partially PEG-protected enzymes are subjected to DHA derivatization, mixed PEG and DHA-protected enzymes are obtained. Examples of various enzyme-DHA derivatized compounds are provided herein:



I

10



wherein $n+m=5$; $m=5$; $n=1-4$ and $m=1-4$; and the symbol ABF^* exemplifies all known forms of the enzyme: ABG, rABG, mABG and mrABG.

An aspartic or glutamic acid free carboxyl group contained in the enzymes is derivatized with diamine spacers attached to DHA providing greater flexibility for the site-specific complex to recognize its binding site, namely the neural membrane.

Other examples are:

$\text{ABG}^*-(\text{CO-S-DHA})_{1-5}$, wherein S is a diamine like spacer ethylene diamine ($\text{NH}_2\text{-CH}_2\text{-CH}_2\text{-NH}_2$) which is the shortest spacer, and

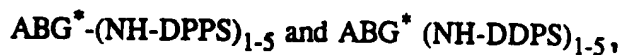


I



The five epsilon amine groups of the enzyme's lysine residues can also be derivatized with DHA-containing phosphatidyl serine, through the serine carboxyl group and the formation of an amide bond (see Table 1). For example:

the symbol ABG^* exemplifies all known forms of the enzyme: ABG, rABG, mABG and mrABG.



wherein DPPS is 2-docosaehaenoyl, 1-palmitoyl-phosphatidylserine, and DDPS is 1,2-didocosaehaenoyl-phosphatidylserine.

Further examples are given with aspartic or glutamic acid residues derivatized with DHA-containing phosphatidyl-ethanolamine:

ABG^{*}-(CO-DPPE)₁₋₅ and ABG-(NH-DDPE)₁₋₅ wherein

DPPE is 2-docosahexaenoyl, 1-palmitoyl-phosphatidylethanolamine and DDPE is 1,2didocosahexaenoyl-phosphatidylethanolamine.

The DHA-containing phospholipids can also form complexes with the various forms of acid beta-glucosidase. Examples are represented below:

$$[\text{ABG}^*]_{1-5} [\text{DPPS}]_{1-5}, \quad [\text{ABG}^*]_{1-5} [\text{DDPS}]_{1-5},$$

$[ABG^*]_{1-5}$ [DPPE] $_{1-5}$ and $[ABG^*]_{1-5}$ [DDPE] $_{1-5}$.

Further, in site-specific biomolecular complexes described below, an epsilon amine group of the enzyme's lysine residue is attached to the carboxyl terminal of the polycationic carrier (polylysine chain:(Lys)₁₋₃₀, and DHA or DHA-containing phospholipid to the amine terminal of the polylysine chain. For example:

(ABG^{*}-NH)-[CO-(Lys)₁₋₃₀-NH]-DHA, (ABG^{*}-NH)-[CO-(Lys)₁₋₃₀-NH]-DPPS and
15 (ABG^{*}-NH)-[CO-Lys)₁₋₃₀-NH]-DDPS.

If DHA derivatization of ABG* is performed only partially, and at least one free epsilon residue is attached to the carboxyl terminal of the polycationic carrier (polylysine:PLL), a new group of complexes are obtained.

$$\begin{array}{ccc}
 \text{ABG}^*-(\text{NH-DHA})_{1-4} & & \text{ABG}^*-(\text{NH-DHA})_4 \\
 | & & | \\
 \text{NH-PLL-DHA,} & & \text{NH-PLL-DPPS}
 \end{array}$$

ABG^{*}-(NH-DHA)₄ wherein **PLL** is **-[CO-(Lys)₁₋₃₀-NH]-**.
I
NH-PLL-DDPS

NH-PLL-DDFS

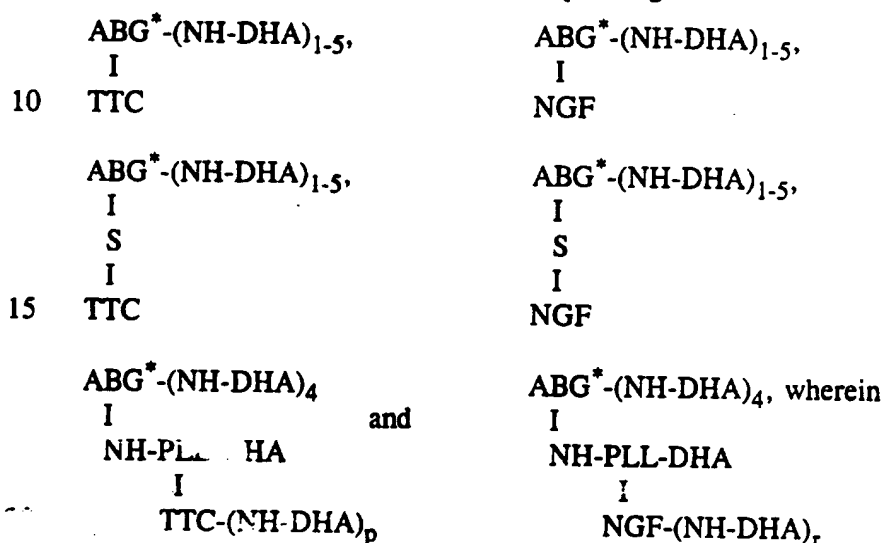
25 A directing moiety such as tetanus toxin fragment C(TTC) or nerve growth factor (NGF) attached to an epsilon amine of the polylysine carrier provide another group of site-specific biomolecular complexes or shuttle vectors with therapeutic uses. The directing moiety is coupled to the docosahexaenoylated enzyme directly or through a diamine spacer. The directing moieties TTC and NGF can themselves be

30 docosahexaenoylated.

-43-

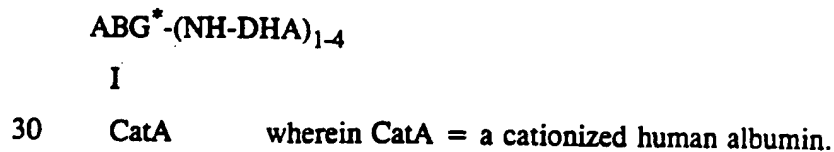
TTC is directive to neurons of the central nervous system and it is internalized by cortical neurons of the gray matter. Thus, it provides the shuttle vector with the additional capability of neuronal endocytosis.

NGF distributes throughout the central nervous system, but it appears to
 5 concentrate in the vicinity of neurons of the cholinergic nervous system including neurons responsible for cognitive and memory processes such as the basal ganglia, the nucleus of Meynart, and the hippocampus in general. Further examples are:



p is the number of epsilon amine groups of lysines in TTC derivatized with DHA and
 r. The number of epsilon amine groups of lysines in NGF derivatized with DHA.

Another directing moiety that is also a cationic carrier is cationized human albumin [Kang, Y.S. and Partridge, W.M., "Brain delivery of biotin bound to a
 25 conjugate of neutral avidin and cationized human albumin," Pharm. Res., 1994, 11(9), 1257-64]. The role of this directing moiety is facilitation of penetration through the blood-brain barrier. As an example is given:



Examples 1 to 13 can be extended by using a wide variety of phospholipids, such as phosphatidyl ethanol amine, acylated phospholipids, lysophospholipids and or

-44-

diacyl glycerol derivatives and phosphatidic acids with the proviso that PUFA chains are in sn2 position or in both sn1 and sn2 position, if they are available.

The amount of different constituents of the vectored transport vehicle (e.g., agent, such as enzyme, proteins, factors, cofactors, hormones, anti-cancer drugs, anti-Parkinson's drugs, etc., carrier, such as PLL of varying molecular weights, different lipophilic residues, and optionally targeting moieties) and all conditions of coupling reactions could be varied so long as the final resultant lipophilic conjugates have a lipophilic-hydrophilic balance within the desired range to assure the transport of the agent intracellularly and controlled release in cell lysosome.

From the preceding description of novel concept of targeting/vectored lipophilic vehicles, conjugates or complexes, and from the above examples, one skilled in the art can easily ascertain the characteristic essential features of this invention, without departing from the invention objectives, and can make various changes in the invention of site-specific biomolecular complex, conjugate or vehicle model to adapt it to various usages and conditions.

Also, those skilled in the art will recognize many equivalents to the specific embodiments of the invention described herein. For example, it is believed that noncovalent bondings could be employed within this invention if the bond strength approached that of the covalent bonds. Accordingly, those equivalents are considered to be part of this invention and are intended to be encompassed by the scope of the following claims.

-45-

WE CLAIM:

1. A site-specific biomolecular complex comprising a biologically active agent and an omega-3 fatty acid and derivatives thereof, with the proviso that said agent cannot be a naturally occurring amino acid.
- 5 2. The complex of claim 1 wherein said complex is held together by van der Waals' and electrostatic forces.
3. The complex of claim 1 wherein said complex is held together by covalent bonds.
4. The complex of claim 1 wherein the omega-3 fatty acid is
10 eicosapentaenoic acid and derivatives thereof.
5. The complex of claim 4 wherein said complex is specific for treating diseases of the glial tissue and cells thereof.
6. The complex of claim 1 wherein the omega-3 fatty acid is docosahexaenoic acid and derivatives thereof.
- 15 7. The complex of claim 6 wherein said complex is specific for treating diseases of cortical, cholinergic and adrenergic neurons.
8. The complex of claim 1 wherein said active agent is selected from the group consisting of peptides, polypeptides, proteins and fragments thereof, fusion proteins, polyethylene glycol-derivatized proteins, enzymes, polyethylene glycol-
20 derivarized enzymes, hormones, growth factors, antigens, antibodies, nucleic acids and derivatives thereof, DNA- and RNA-segments, oligonucleotides, antisense oligonucleotide analogs, nucleotides and derivatives thereof, genes, transfection vectors, expression vectors, cloning vectors, drugs and diagnos agents.

9. The complex of claim 8, wherein the enzyme is selected from a group consisting of glucocerebrosidase, acid beta glucosidase, alglucerase, cerezyme and ceredase and derivatives thereof.
10. The complex of claim 9, wherein the said enzymes have been derivatized with polyethylene glycol.
11. The complex of claim 8 further comprising a blood-brain barrier permeabilizer peptide.
12. The complex of claim 1 further comprising a cationic carrier.
13. A complex according to claim 12 wherein said carrier is a poly amino acid selected from the group consisting of poly-lysine, poly-arginine and poly-ornithine.
14. The complex of claim 13 wherein said poly amino acid is a poly-lysine having 1-50 lysine residues.
15. The complex of claim 14 wherein said poly-lysine has 10 to 25 lysine residues.
16. The complex of claim 1 further comprising a targeting moiety.
17. The complex of claim 8 further comprising a targeting moiety.
18. The complex of claim 9 further comprising a targeting moiety and a cationic carrier.
19. The complex of claim 10 further comprising a targeting moiety and a cationic carrier.

-47-

20. The complex of claim 12 further comprising a targeting moiety.
21. The complex of claim 16 wherein said targeting moiety is selected from the group consisting of C fragment of tetanus toxin, alpha-bungarotoxin and nerve growth factor.
- 5 22. The complex of claim 20 wherein said targeting moiety is selected from the group consisting of C fragment of tetanus toxin, alpha-bungarotoxin and nerve growth factor.
23. The complex of claim 22 wherein said complex comprises an enzyme selected from a group consisting of ceramidase, alpha-galactosidase,
10 glucocerebrosidase, beta-galactosidase, beta-hexosaminidase A, beta-hexosaminidase B, galactosylceramidase, arylsulfatase A, ganglioside sialidase, sphingomyelinase, alpha-N-acetylgalactosaminidase, aspartylglycosaminidase, alpha-L-fucosidase, sialidase, alpha-mannosidase, beta-mannosidase, iduronate sulfatase, alpha-L-iduronidase, GalNAc 4-sulfatase, arylsulfatase B, Gal 6-sulfatase, heparan N-
15 sulfatase, alpha-N-acetylglucosaminidase, AcetylCoA: glucosamine-acetyltransferase, GlcNAc-6-sulfatase, beta-glucuronidase, alpha-glucosidase, acid lipase and 6-phospho-N-acetylglucosamine transferase.
24. A method for the site-directed delivery of active agents to mammals afflicted with Gaucher's disease, which method comprises administering to said
20 mammals a therapeutically effective amount of a complex of claim 9.
25. A method for the treatment of Gaucher's disease, which method comprises administering to said afflicted mammals a therapeutically effective amount of a complex of claim 10 effective in treating said disease.
26. A method of claim 23, wherein said complex is a complex of claim 18.

27. A method of claim 24, wherein said complex is a complex of claim 19.
28. A method of claim 24, wherein said complex is a complex of claim 22.
29. The method of claim 27 wherein the administration is done by a method selected from the group consisting of intrathecal injection, surgical implant, direct
5 interstitial infusion into the brain and intravenous infusion.
30. A method for the site-directed delivery of biologically active agents to treat diseases of the glial tissue of a mammalian brain, which method comprises administering to said afflicted mammal a therapeutically effective amount of a site-specific biomolecular complex comprising a biologically active agent and
10 eicosapentaenoic acid and derivatives thereof.
31. A method for the treatment of glial cancers, which method comprises administering to said afflicted mammals a therapeutically effective amount of a site-specific biomolecular complex comprising a biologically active agent and an omega-3 fatty acid and derivatives thereof.
- 15 32. A method for the site-directed delivery of biologically active agents to treat diseases of the cortical, cholinergic and adrenergic neurons of a mammalian brain, which method comprises administering to said afflicted mammal an effective amount of a site-specific biomolecular complex comprising a biologically active agent and docosahexaenoic acid and derivatives thereof.
- 20 33. The method of claim 30, 31 and 32 wherein said biologically active agent is selected from the group consisting of peptides, polypeptides, proteins and fragments thereof, fusion proteins, polyethylene glycol-derivatized proteins, enzymes, hormones, growth factors, antigens, antibodies, nucleic acids and derivatives thereof, DNA- and RNA-segments, oligonucleotides, antisense oligonucleotide analogs,

-49-

nucleotides and derivatives thereof, genes, transfection vectors, cloning vectors, drugs and diagnostic agents.

34. The method of claim 30 wherein said complex further comprises an additional moiety selected from the group consisting of permeabilizer peptides and cationic carriers selected from the group consisting of poly-lysine, poly-arginine and poly-omithine and mixtures of said peptides and said polycationic carriers.

35. The method of claim 31 wherein said complex further comprises an additional moiety selected from the group consisting of permeabilizer peptides and cationic carriers selected from the group consisting of poly-lysine, poly-arginine and poly-omithine and mixtures of said peptides and said polycationic carriers.

36. The method of claim 32 wherein said complex further comprises an additional moiety selected from the group consisting of permeabilizer peptides and cationic carriers selected from the group consisting of poly-lysine, poly-arginine and poly-omithine and mixtures of said peptides and said polycationic carriers.

37. The method of claim 32 wherein said complex further comprises a targeting moiety selected from the group consisting of C fragment of tetanus toxin, alpha-bungarotoxin and nerve growth factor.

38. The method of claim 36 wherein said complex further comprises a targeting moiety selected from the group consisting of C fragment of tetanus toxin, alpha-bungarotoxin and nerve growth factor.

39. The method of claims 30, 31 and 32 wherein said administration is done by a method selected from the group consisting of intrathecal injection, surgical implant or direct interstitial infusion into the brain, intravenous and oral administration.

-50-

40. The method of claim 36, 37 and 38, wherein said administration is done by a method selected from the group consisting of intrathecal injection, surgical implant, direct interstitial infusion into the brain, intravenous and oral administration.

1/7

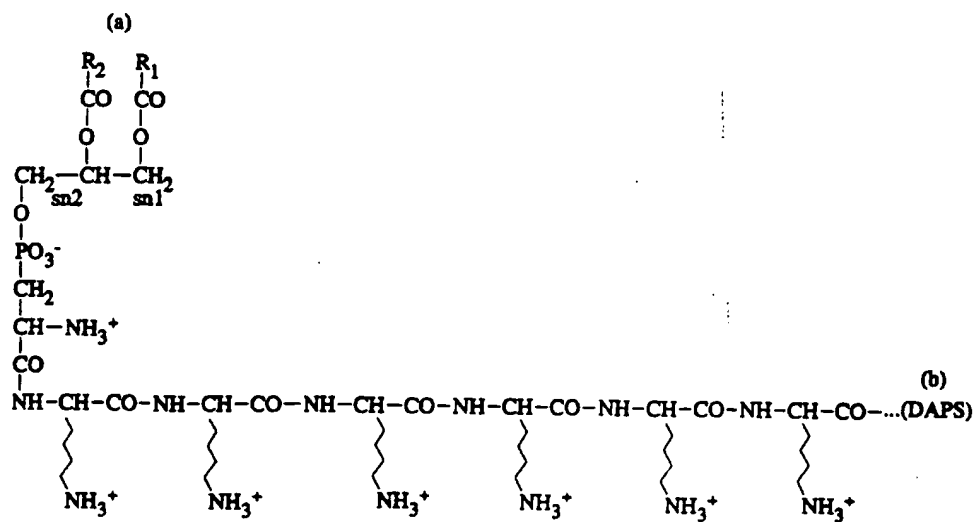


Fig. 1

2/7

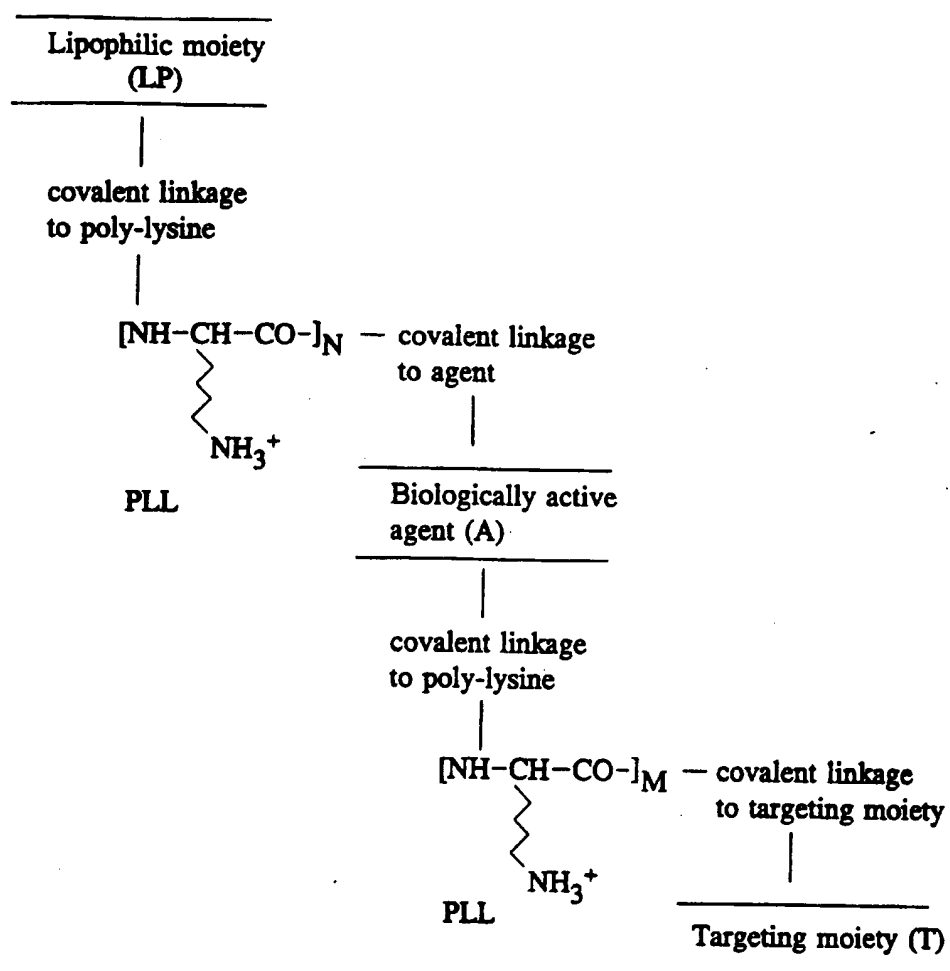


Fig. 2

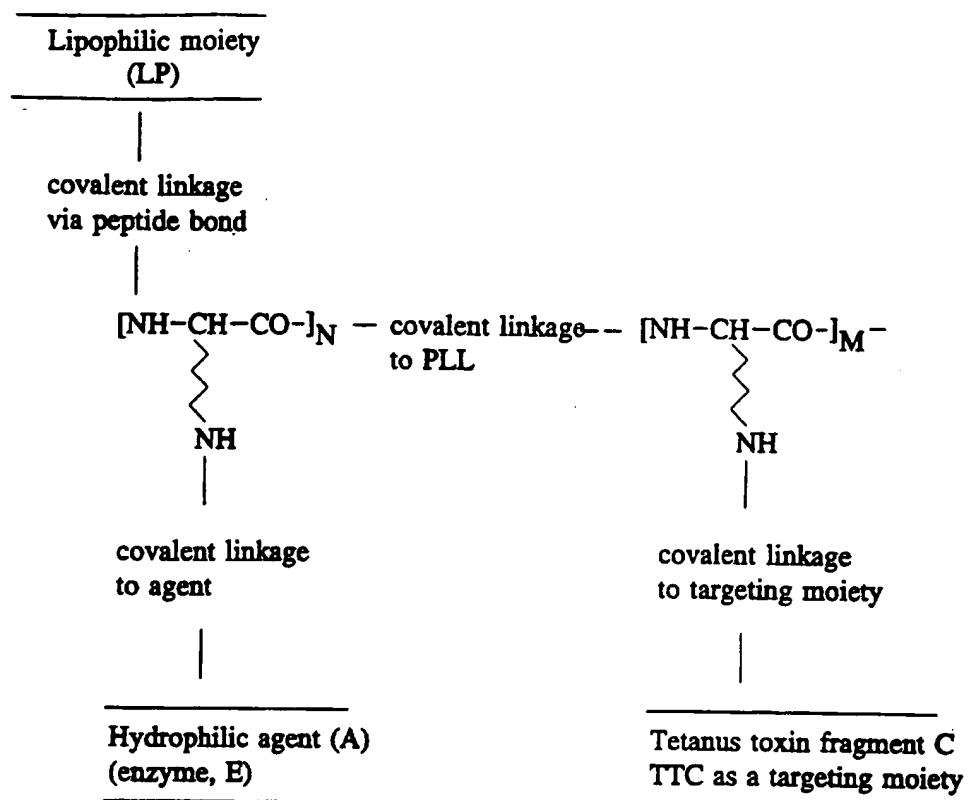


Fig. 3

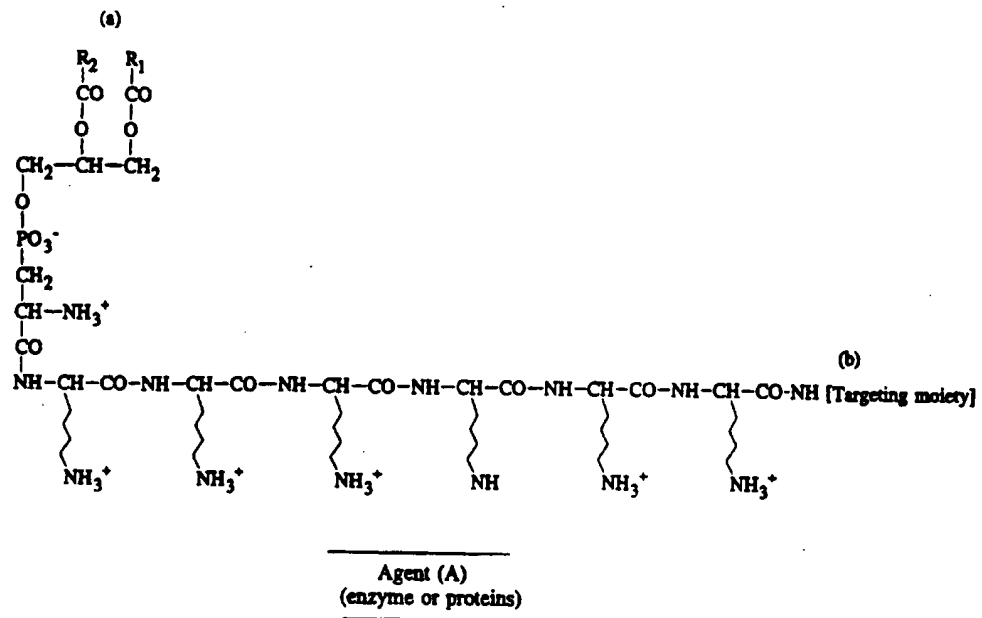


Fig. 4

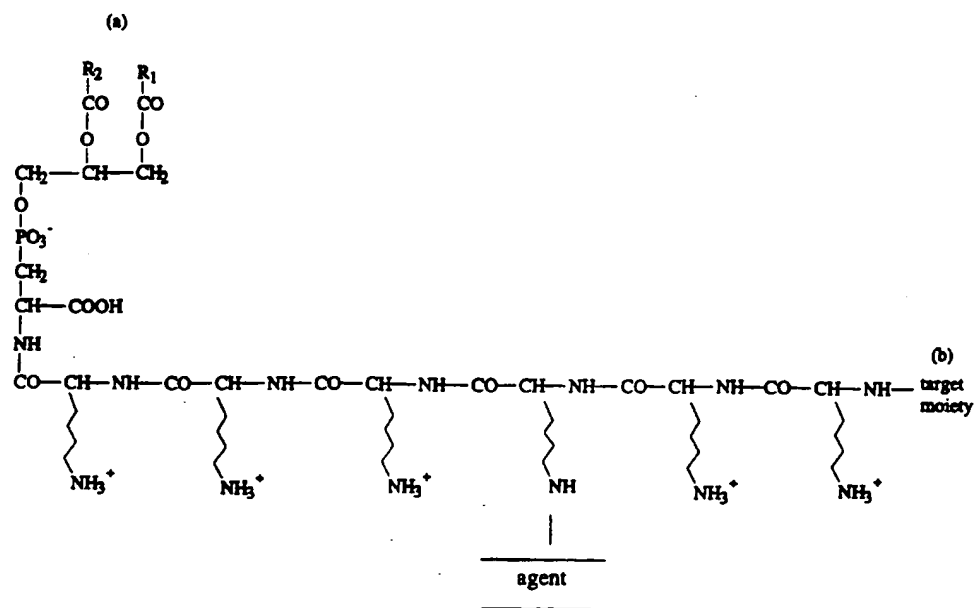


Fig. 5

(a)

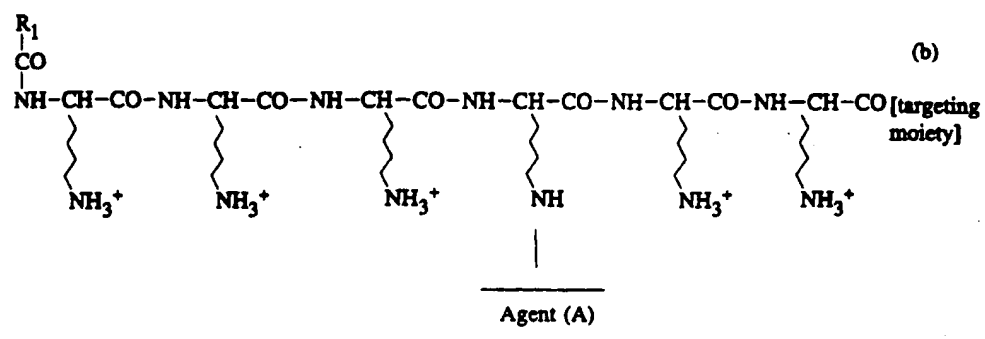


Fig. 7

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/09870

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 38/00, 38/18, 31/70, 39/44, 39/385, 39/395

US CL : 514/2, 44; 424/130.1, 179.1, 181.1, 193.1, 195.11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAPlus, Registry, BIOSIS, Medline, EMBASE, WPIDS

Search terms: fatty acid, omega-3, docosahexaenoic, eicosapentaenoic, conjugate, complex, brain, glial, Gaucher's

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO, A, 92/20362 (SHASHOUA) 26 November 1992, see entire document.	1, 2, 6-8, 16, 17, 21, 22 ----- 3-5, 9-15, 18-20, 23-25, 30-40
Y	Neuroscience Letters, Volume 171, issued 1994, M. Alberghina et al., "Differential transport of docosahexaenoate and palmitate through the blood-retina and blood-brain barrier of the rat", pages 133-136, see entire document.	1-25, 30-40

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

Special categories of cited documents:	
A document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
E earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means	*A* document member of the same patent family
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

07 NOVEMBER 1995

Date of mailing of the international search report

11 DEC 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

Bequeline Krikorian

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/09870

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Clinical and Experimental Metastasis, Volume 11, Number 2, issued 1993, T. Narai et al., "Intravenously injected radiolabelled fatty acids image brain tumour phospholipids in vivo: differential uptakes of palmitate, arachidonate and docosahexaenoate", pages 141-149, see entire document.	1-25, 30-40

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/09870

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 26
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 26 refers to the method of claim 23. However, claim 23 is not drawn to a method.

3. ☒ Claims Nos.: 26-29
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/09870

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I. Claims 1-24, drawn to a site-specific bimolecular complex comprising a biologically active agent and an omega-3 fatty acid and derivatives thereof and a first method of use.

Group II. Claim 25, drawn to a method for the treatment of Gaucher's disease.

Group III. Claims 30, 33 (in part), 34, and 39 (in part), drawn to a method for the site-directed delivery of active agents to treat diseases of the glial tissue of a mammalian brain.

Group IV. Claims 31, 33 (in part), 35, and 39 (in part), drawn to a method for the treatment of glial cancers.

Group V. Claims 32, 33 (in part), 36-38, 39 (in part), and 40, drawn to a method for the site-directed delivery of active agents to treat diseases of the cortical, cholinergic, and adrenergic neurons of a mammalian brain.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The bimolecular complex of Group I can be used in a materially different manner from that claimed, such as to regulate the growth of cells in vitro, for example. The methods of Groups II-V are different because they involve different objectives, issues, and steps.

THIS PAGE BLANK (USPTO)